Genet Resour Crop Evol (2008) 55:105–114
DOI 10.1007/s10722-007-9218-5

RESEARCH ARTICLE

Microsatellite markers and genetic diversity assessment in *Lolium temulentum*

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Received: 10 August 2006 / Accepted: 29 January 2007 / Published online: 18 April 2007

Abstract *Lolium* and *Festuca* are two important genera of cool-season forage and turf grasses worldwide. *Lolium temulentum* L. (darnel ryegrass) has been proposed as a model species for genomics studies of cool-season forage and turf grasses. A study with 41 darnel ryegrass, three tall fescue (*Festuca arundinacea* Schreb.), two tetraploid fescue (*F. glaucescens*), and two meadow fescue (*F. pratensis*) genotypes was initiated to (i) identify a set of microsatellite (simple sequence repeats) markers useful for *L. temulentum* L., and (ii) to utilize such markers for assessing the genetic variability of *L. temulentum* accessions collected from different geographical regions of the world. A total of 40 tall fescue (TF) EST-SSRs and 60 *Festuca–Lolium* (F × L) genomic SSRs were screened on a subset of eight genotypes. The selected 30 tall fescue EST-SSRs and 32 F × L genomic SSRs were used for further analysis of genotypes. The TF-EST- and the F × L genomic-SSRs identified 10.3 and 9.3 alleles per marker, respectively with an average polymorphic information content (PIC) value of 0.66. The phenogram based on 319 EST-SSR and 296 genomic SSR fragments, grouped *L. temulentum* accessions into three major clusters except for accession ABY-BA 8892.78. *Lolium temulentum* accession ABY-BA 8892.78 did not cluster with any other accession. The *Festuca* clusters were distantly related with darnel ryegrass clusters with a similarity coefficient of 0.26. The selected set of tall fescue EST- and F × L genomic SSRs were useful in assessing *L. temulentum* genetic diversity and could benefit the genetic improvement of members of the *Festuca–Lolium* complex.

Keywords *Festuca* spp. · Genetic diversity · *Lolium temulentum* · Microsatellite markers

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Introduction

Members of the *Festuca–Lolium* complex are widely used as forage and turf, especially in the temperate regions of the world. They contain highly productive forage grass species that render numerous benefits to humans, including providing feed and fodder for millions of dairy and beef cattle, horses, sheep, and many wild animals (Wang et al. 2002). These grasses also play a major role as turf in golf courses and

lawns worldwide. These grasses belong to the subfamily Pooideae and tribe Poeae. Among these grasses, tall fescue (*Festuca arundinacea* Schreb.) is an allohexaploid (2n = 6x = 42) with three genomes, PG1G2 (Seal 1983). Other cultivated cool season grasses in the genus *Festuca* include tetraploid fescue (*Festuca glaucescens* Hegetschw. & Heer., the donor of the G1 and G2 genomes) and meadow fescue (*Festuca pratensis* (Hudson) P. Beauv., the donor of the P genome) (Sleper 1985). The genus *Lolium* is comprised of the outcrossing *Lolium perenne* L., *Lolium multiflorum* Lam., the self-pollinated *L. temulentum* L. subsp. *temulentum* (darnel, darnel ryegrass), and *L. persicum* Boiss. et Hohen. ex Boiss. (Persian darnel). It has been demonstrated that *L. temulentum* is closely related to other members of the *Festuca–Lolium* complex as well as a number of other important forage and turf grass species (Mian et al. 2005).

Darnel ryegrass is widely distributed throughout the world. It is found growing as mimic weed with similar life cycle and morphology in wheat and barley fields. It is a long day annual grass that is classified as a noxious weed in Arkansas and as a plant pest in South Carolina (Kuk et al. 2000). However, because of the simplicity of the biology of darnel ryegrass, it is being used as a model species for genetic and genomic studies in forage and turf grasses (Wang et al. 2005). As a model species for forage and turf grasses, darnel ryegrass offers the following advantages: it is self-fertile, has a short life cycle (less than 3 months), is a diploid, is easy to grow, and closely related to major grass species in the *Festuca–Lolium* complex and others. In contrast, most important forage and turf grasses require vernalization to flower, many are polyploids, and are obligate outbreeders with gametophytic self incompatibility systems largely controlled by the SZ multi- allelic locus (Lundqvist 1962; Fearon et al. 1983).

Interspecific hybridization between species in the *Festuca–Lolium* complex has been exploited in the development of forage germplasm of high-quality and winter hardiness, and in the introgression of abiotic stress tolerance traits (Humphreys et al. 2005). Members of the *Lolium* genus are high yielding and produce fodder of high quality and digestibility. On the other hand, members of the genus *Festuca* have high resistance to abiotic stresses which include winter hardiness, drought resistance and persistence. Moreover, biotic stress genes have been introgressed from *Festuca* to ryegrass species (Armstead et al. 2006). Combining the attributes of both genera is feasible through hybridization because chromosomes of the two have high homology, hybridize and have a high frequency of recombination resulting in fertile offspring (Jauhar 1993; Zwierzykowski et al. 1999; Terrel 1966). This is possible because hybrids between the two undergo promiscuous chromosome recombination which enables gene transfer from one homoeologous chromosomal region to another (Humphreys and Pašakinskienė 1996; Humphreys et al. 2003). Furthermore, introgressed segments of chromosomes from one species through interspecific hybridization can be distinguished by genomic *in situ* hybridization (GISH) (Thomas et al. 2003). Intergeneric hybridization between *Lolium* × *Festuca* can be used for the production of androgenic plants that may display transgressive resistance for abiotic stresses (Humphreys et al. 2003).

Simple sequence repeats (SSRs) (Microsatellite) are among the most variable DNA sequences. They are highly polymorphic, abundant, easy to use, and have become an important marker system in cultivar fingerprinting, diversity studies, molecular mapping and in marker-assisted selection (Goldstein and Schlöterer 1999). The loci of these markers are highly transferable across species (>50%) within a genus (Röder et al. 1995; Peakall 1998; Thiel et al. 2003; Saha et al. 2004; Eujayl et al. 2004), but that transferability is low across genera (Peakall 1998; Roa et al. 2000; Thiel et al. 2003). However, SSR markers derived from expressed sequence tags (EST-SSRs) are likely to be more transferable because they are a part of the transcribed regions of DNA (Scott et al. 2000). Transcribed regions are more conserved across species and genera and EST-SSRs can be used for comparative mapping (Saha et al. 2004; Yu et al. 2004).

Darnel ryegrass has been used as a model in the study of a senescence- induced degradation gene in *F. pratensis* through introgression into *L. temulentum* background (Thomas et al. 1999). In addition to its demonstrated potential as a model species for a number of important forage and turf grass species, darnel ryegrass can also be exploited for introgression of its self-compatibility genes into the out-crossing members of the *Festuca–Lolium* complex (Yamada 2001). Understanding the genetic diversity among

accessions and cultivars within a species is important for their efficient use in genetic and genomic manipulation studies. The Noble Foundation maintains a large world-wide collection of darnel ryegrass accessions and the genetic relatedness among these accessions has not been reported. At present, only few SSR markers are available for darnel ryegrass (Senda et al. 2003). Potential use of tall fescue EST-SSR markers developed at the Noble Foundation for genetic studies in darnel ryegrass has been demonstrated (Wang et al. 2005; Mian et al. 2005). Identification of a large number of cross-species microsatellite markers would be useful for genetic characterization of darnel ryegrass. The objectives of this study were: (a) to identify a set of cross-species SSR markers useful for study of *L. temulentum* and (b) to assess the genetic diversity among the *L. temulentum* accessions at the Noble Foundation.

Materials and methods

Plant materials

The 41-darnel ryegrass accessions used in this study were mostly plant introductions (PI) from 17 different countries (Table 1). Seeds of these accessions are maintained in our cold seed storage. Seeds were germinated in petri-dishes and later transplanted to 2 × 2 inch pots. Additionally, three tall fescue genotypes, two tetraploid fescue genotypes, and two diploid fescue genotypes were used. The fescue plants were split from clones and planted in 2 × 2 inch pots. Leaf tissues were collected and frozen in liquid nitrogen from vigorously growing seedlings/saplings after establishment in the greenhouse. Tissue was collected from three and 10 plants from each *Lolium* accession and fescue genotype, respectively.

DNA extraction

Approximately 200 mg of leaf tissue from each accession/genotype was taken in a 2.0 ml microcentrifuge tubes and ground to fine powder using a mixer mill (Retsch, Hannover, Germany). DNA was extracted from ground tissue using the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). DNA concentration was determined using a HOEFER Dyna Quant 200 (Hoefer, Inc., San Francisco, CA).

Molecular markers and amplification

A total of 40 tall fescue EST-SSRs (TF EST SSRs from Saha et al. 2004) and 60 *Festuca–Lolium* (F × L) genomic SSRs (kindly provided by Dr. Marc Ghesquiere, Lusignan, INRA, France) were screened with a subset of 8 genotypes (L6, L7, L18, L25, L30, L39, PI and PI383647) (Table 1). Thirty TF EST SSRs and 32 F × L genomic SSRs with clean amplification products were selected for further analysis with all genotypes. The PCR reactions were run under standard conditions for all primers using 1 U AmpliTaq Gold with GeneAmp PCR buffer II (Applied Biosystems/Roche, Branchburg, NJ), 3 mM $MgCl_2$, 200 μM dNTPs, 0.2 mM of each primer and 20 ng of template DNA in a 10 μl reaction. Thermocycler model ABI 9700 was used for PCR amplification and the reaction conditions consisted of 10 min at 95°C, followed by 40 cycles of 50 s at 95°C, 50 s at a temperature between 58°C and 64°C (optimum annealing temperature for each primer pair), 90 s at 72°C, and a final extension step of 10 min at 72°C.

Data acquisition and analysis

The PCR products were resolved using an ABI3730 according to standard protocol (Schuelke 2000). GeneScan™ files were imported into GenoGrapher software (GenoGrapher v1.6) for gel images and thumbnails and subsequently used to score SSR bands as present or absent. Polymorphism information content (PIC), a measure of allelic diversity at a given locus, was calculated as follows: $PIC = 1-\sum f^2_i$, where f_i is the frequency of the ith allele (Weir 1990). Two PIC values were calculated, one for all genotypes and one for *Lolium* genotypes only.

Similarity matrices for the genotypes were calculated using NTSYS-PC 2.10 (Applied Biostatistics, Setauket, New York, USA). The F × L genomic SSRs and the TF EST SSRs were analyzed separately to construct the matrices and then the two data sets were combined and re-analyzed. The genetic similarity among genotypes was calculated by procedure SIMQUAL using the DICE similarity coefficient (Dice, 1945). This measure of similarity is the same as Nei and Li's (1979) similarity coefficients. For clustering, the SAHN (Sequential Agglomerative Hierarchical and Nested Clustering) (Sneath and Sokal 1973) was

Table 1 *Lolium* and *Festuca* accessions used in the study, organized by country of origin and plant species

Lab ID	Donor/collector	Scientific name	Cultivar/PI	Origin
L31	WRPIS	*Lolium temulentum*	PI 219925	Afghanistan
L43	WRPIS	*Lolium temulentum*	PI 220103	Afghanistan
L44	WRPIS	*Lolium temulentum*	PI 220807	Afghanistan
L21	IGER	*Lolium temulentum*	ABY-BA 13197.00	Australia
L22	IGER	*Lolium temulentum*	ABY-BA 13198.00	Australia
L23	CSIRO	*Lolium temulentum*	Lt Ceres	Canada
L27	WRPIS	*Lolium temulentum*	PI 195000	Ethiopia
L28	WRPIS	*Lolium temulentum*	PI 196866	Ethiopia
L42	WRPIS	*Lolium temulentum*	PI 197439	Ethiopia
L6	IGER	*Lolium temulentum*	ABY-BA 8892.78	France
L11	IGER	*Lolium temulentum*	ABY-BA 10918.95	France
L5	IGER	*Lolium temulentum*	ABY-BA 8474.95	Germany
L48	WRPIS	*Lolium temulentum*	PI 477121	Germany
L9	IGER	*Lolium temulentum*	ABY-BA 9778.00	Greece
L10	IGER	*Lolium temulentum*	ABY-BA 10332.95	Greece
L13	IGER	*Lolium temulentum*	ABY-BA 11129.95	Greece
L46	WRPIS	*Lolium temulentum*	PI 302664	India
L25	WRPIS	*Lolium temulentum*	Lt 165903	India
L41	WRPIS	*Lolium temulentum*	PI 166370	India
L1	IGER	*Lolium temulentum*	ABY-BA 6777.95	Iran
L7	IGER	*Lolium temulentum*	ABY-BA 8917.78	Iran
L12	IGER	*Lolium temulentum*	ABY-BA 11128.98	Italy
L18	IGER	*Lolium temulentum*	ABY-BA 12284.00	Italy
L32	WRPIS	*Lolium temulentum*	PI 239799	Italy
L45	WRPIS	*Lolium temulentum*	PI 239801	Italy
L14	IGER	*Lolium temulentum*	ABY-BA 11306.95	Morocco
L17	IGER	*Lolium temulentum*	ABY-BA 12214.98	Morocco
L47	WRPIS	*Lolium temulentum*	PI 422589	Morocco
L35	WRPIS	*Lolium temulentum*	PI 391427	Morocco
L30	WRPIS	*Lolium temulentum*	PI 219594	Pakistan
L29	WRPIS	*Lolium temulentum*	PI 218085	Pakistan
L15	IGER	*Lolium temulentum*	ABY-BA 12091.93	Portugal
L19	IGER	*Lolium temulentum*	ABY-BA 13138.96	Portugal
L20	IGER	*Lolium temulentum*	ABY-BA 13157.00	Portugal
L16	IGER	*Lolium temulentum*	ABY-BA 12188.98	Spain
L34	WRPIS	*Lolium temulentum*	PI 287848	Spain
L36	WRPIS	*Lolium temulentum*	PI 415813	Switzerland
L8	IGER	*Lolium temulentum*	ABY-BA 9169.98	Tunisia
L26	WRPIS	*Lolium temulentum*	PI 180449	Turkey
L39	AARI	*Lolium temulentum*	TR 12099	Turkey
L40	AARI	*Lolium temulentum*	TR 47251	Turkey
MF316	WRPIS	*Festuca pratensis*	PI283316	Turkey
MF647	WRPIS	*Festuca pratensis*	PI383647	Spain
TetFW1	UK	*Festuca glaucescens*	W1	USA

Table 1 Continued

Lab ID	Donor/collector	Scientific name	Cultivar/PI	Origin
TetFPI	UK	*Festuca glaucescens*	PI	USA
TF901	WRPIS	*Festuca arundinacea*	PI297901	Australia
TF078	WRPIS	*Festuca arundinacea*	PI423078	Spain
TKY31	UK	*Festuca arundinacea*	Kentucky 31 (3–11)	USA

WRPIS: Western Regional Plant Introductions Station, USA; IGER: Institute of Grassland and Environmental Research, Wales; CSIRO: Commonwealth Scientific and Industrial Research Organization, Australia; AARI: Aegean Agricultural Research Institute, Turkey; UK: University of Kentucky, USA

used to construct phenograms using the similarity coefficients. The 'TM' option was set to 'FIND' to detect all possible trees using the UPGMA method (un-weighted pair group method with arithmetic mean). The TREE procedure was used to create the phenogram. Bootstrap analysis was performed using the program PAUP* 4.0 beta (Swofford 2002) with 1000 replications following the UPGMA algorithm. Trees were retained if they appeared at least 80% of the time.

Evaluation of *L. temulentum* accession L6

Seeds of nine accessions of *L. temulentum* were randomly selected from each of the major clusters and sub-clusters along with L6 and compared with meadow fescue. Ten seeds of each accession were measured for length and width and averaged to indicate the seed size. Weights of 100 seeds were measured using a digital electric balance with four decimal points. For morphological evaluation, seeds were planted in 3 cm^2 flats, grown in greenhouse and evaluated 35 days after planting.

Results and discussion

SSR marker development

A total of 30 TF EST-SSRs were selected based on clean amplification products from the 40 TF SSRs initially screened. Similarly, 32 F × L genomic SSRs were selected from the 60 initially screened. This translates into a transfer rate of 71 and 53% for the TF SSRs and F × L genomic SSRs, respectively. Although the number of SSRs screened was different (40 versus 60), the results imply that the EST SSRs had a higher rate of transferability than the genomic SSRs. Wang et al. (2005) reported differences in transfer rates between genomic and EST SSRs where genomic SSRs detected higher numbers of alleles per maker loci. EST-SSRs are derived from transcribed regions of DNA and therefore expected to be more conserved and transferable across genera (>50%) relative to those derived from genomic regions (Röder et al. 1995; Peakall 1998; Scott et al. 2000; Thiel et al. 2003; Saha et al. 2004; Eujayl et al. 2004). Higher genomic SSR polymorphism has been reported in other studies on germplasm characterization and genetic diversity (Varshney et al. 2005).

SSR amplification and polymorphism

Two hundred and ninety six fragments were detected among 41 accessions of *L. temulentum*, 3 of tall fescue, 2 of meadow fescue and 2 of tetraploid fescue for 32 F × L genomic SSRs while 319 were detected for 30 TF EST-SSRs (Tables 1, 2). The sizes of the bands ranged from 68 to 496 bp for both sets of markers. The total number of bands per SSR primer pair was 9.3 and 10.6 for the F × L genomic SSRs and the TF EST-SSRs, respectively. In this study, the average number of bands per SSR was about the same for the EST- and the genomic SSRs. This may be explained by the nature and attributes of the chromosomes of the two genera. The chromosomes of *Festuca* and *Lolium* have high homology and chromosomes from the two hybridize, when crossed, resulting in fertile offspring (Jauhar 1993; Zwierzykowski et al.1999; Terrel 1966). In meiosis, hybrids undergo promiscuous chromosome recombination and gene transfer from one homoeologous chromosome region to another occurs (Humphreys and Pašakinskienė 1996; Humphreys et al. 2003). Therefore, F × L hybrids from which genomic SSRs were derived contained genetic material from both species.

Table 2 *Festuca* × *Lolium* genomic SSRs and tall fescue EST-SSRs with their corresponding number of bands and polymorphic information content (PIC) for all accessions/genotypes and *Lolium* accessions only

Festuca × *Lolium* genomic SSRs				Tall fescue EST-SSRS			
Marker	Number	PIC	PIC	Marker	Number	PIC	PIC
Name	Bands	All	*Lolium*	Name	Bands	All	*Lolium*
B1-A11	11	0.72	0.44	NFFA013	19	0.89	0.87
B1-A2	17	0.92	0.86	NFFA015	12	0.81	0.72
B1-A8	12	0.83	0.74	NFFA017	14	0.66	0.61
B1-A9	5	0.68	0.63	NFFA019	12	0.78	0.73
B1-B2	5	0.37	0.05	NFFA024	9	0.38	0.13
B1-B3	5	0.41	0.09	NFFA029	6	0.47	0.05
B1-B6	7	0.43	0.14	NFFA031	9	0.58	0.17
B1-C1	15	0.74	0.60	NFFA032	8	0.69	0.53
B1-C8	10	0.77	0.73	NFFA033	8	0.46	0.00
B1-C9	10	0.68	0.67	NFFA034	5	0.25	0.64
B2-B7	6	0.76	0.75	NFFA036	7	0.60	0.16
B2-D1	10	0.83	0.84	NFFA041	4	0.59	0.49
B2-D11	3	0.52	0.50	NFFA047	7	0.22	0.00
B2-D8	2	0.50	0.49	NFFA048	15	0.70	0.51
B2-F3	11	0.43	0.13	NFFA057	11	0.81	0.77
B2-G3	14	0.90	0.89	NFFA059	16	0.83	0.68
B2-G6	26	0.90	0.83	NFFA061	17	0.85	0.82
B3-A3	4	0.36	0.09	NFFA066	12	0.75	0.68
B3-A4	5	0.64	0.60	NFFA069	8	0.70	0.64
B3-B1	7	0.39	0.05	NFFA071	9	0.78	0.64
B3-B6	5	0.61	0.57	NFFA073	6	0.35	0.10
B3-B8	11	0.84	0.59	NFFA075	10	0.60	0.28
B3-C10	7	0.68	0.42	NFFA096	7	0.46	0.00
B3-C11	10	0.57	0.20	NFFA098	19	0.87	0.86
B3-C4	4	0.42	0.30	NFFA100	18	0.76	0.71
B3-C5	13	0.82	0.76	NFFA103	12	0.78	0.69
B3-D12	16	0.81	0.72	NFFA120	5	0.67	0.68
B3-D2	13	0.88	0.82	NFFA126	7	0.82	0.82
B3-D3	10	0.81	0.75	NFFA135	15	0.88	0.86
B3-F3	5	0.63	0.31	NFFA142	12	0.74	0.58
B4-C3	10	0.74	0.68				
B4-C4	7	0.49	0.42				
Mean	9.25	0.66	0.52		10.63	0.66	0.51
Std. dev.	5.00	0.18	0.27		4.37	0.19	0.30

The polymorphic information content (PIC) values were calculated based on either all 48 genotypes tested or only on the basis *Lolium* genotypes for the F × L genomic- and TF EST-SSRs (Table 2). The PIC is an estimate of the ability of a marker to differentiate genotypes based on both the number of alleles expressed and their relative frequencies (Weir 1990). PIC values for the two maker sources were similar where F × L genomic SSRs ranged from 0.36 to 0.92 based on all genotypes and from 0.05 to 0.89 based on *Lolium* accessions only. PIC for TF EST-SSRs ranged from 0.22 to 0.89 and from 0.0 to 0.87 for all

genotypes and for *Lolium* accessions, respectively. Both marker sources had similar mean PIC values based on all genotypes (0.66).

Some SSR markers had lower PIC values on *Lolium* accessions than on all genotypes e.g. B1-B2, B3-B1, B3-A3 and B1-B3 for F × L genomic SSRs and NFFA047, NFFA073, NFFA033 and NFFA096 for the TF EST-SSRs. On the other hand, some SSRs had high PIC values in both *Lolium* accessions and all genotypes e.g. B2-D1, B3-D2, B2-G6, B1-A2 and B2-G3 for F × L genomic SSRs and NFFA126, NFFA061, NFFA098, NFFA135 and NFFA013 for the TF EST-SSRs. The similarity of mean PIC scores for both marker systems implies that the two marker sources had similar power to differentiate the accessions tested. The PIC values depend on the type of marker (whether dominant or codominant) and on the number and frequency of alleles per marker locus. The SSR loci are multi allelic and codominant and therefore expected to have high PIC values. However, EST SSR markers were reported to have lower levels of informativeness (low PIC) relative to genomic SSRs (Cho et al. 2000; Eujayl et al. 2002). The similarity of mean PIC values between genomic and EST SSRs used in this study implies that these ESTs are highly informative (Saha et al. 2004; Varshney et al. 2005).

Genetic similarity in *L. temulentum*

The TF EST-SSRs, F × L genomic SSRs, and combined data all revealed similar major clusters with only minor changes within clusters, thus only the tree based on combined data is presented in Fig. 1. The 41 *L. temulentum* accessions formed 3 clusters (L1–L28, L7–L32, and L25–L45) where the first cluster had 3 distinct sub-clusters (L1–L5, L8–L41and L27–L28) (Fig. 1). Accession L6 (ABY-BA 8892.78) did not cluster with any other accession. The Meadow-, tetraploid- and tall fescues each formed a distinct cluster.

L6 (ABY-BA 8892.78) was genetically distinct from the other 40 *L. temulentum* accessions. This accession grouped more closely with the meadow

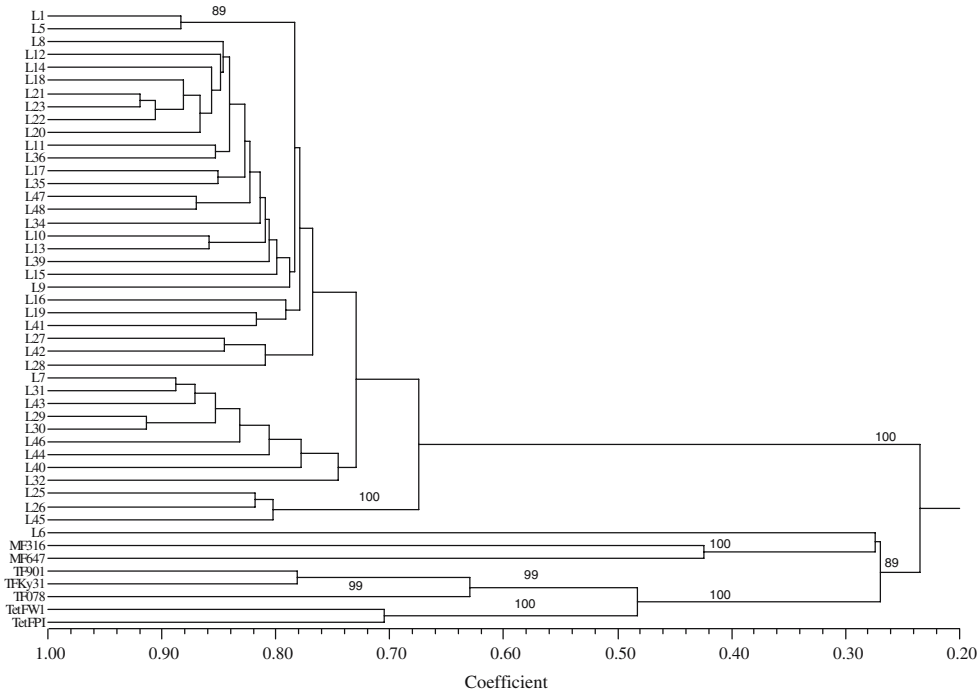

Fig. 1 A phenogram of 41 *Lolium temulentum* (L), three tall fescue (*Festuca arundinacea*) (TF), two tetraploid fescue (*F. glaucescens*) (Tet), and two diploid fescue (*F. pratensis*) (MF) accessions constructed based on DICE similarity coefficients calculated from both EST- and *Festuca–Lolium* genomic SSR marker data. Thirty EST- SSRs and 32 *Festuca–Lolium* genomic SSRs giving 319 and 296 fragments, respectively, were used. The numbers in figure are bootstrap values after 1000 replications

fescue accessions than with *Lolium* accessions. Thus L6 appears to be a unique accession that is genetically very different from all other *L. temulentum* accessions in this study. Molecular markers distinguish accessions based on differences in their DNA sequence and not on appearance or morphology. The microsatellite markers discriminate the genotypes into distinct classes and information gained from genetic diversity studies can play important role in incorporating *L. temulentum* traits into breeding programs of other related species.

In terms of the origin of the accessions, the first cluster (L1–L28) was composed of 28 mainly European- African accessions. One sub cluster (L27, L28 and L42) in this cluster was composed of three accessions from Ethiopia. All other clusters were composed of accessions from different countries and ecoregions except for the tetraploid fescues that are from the USA. Using both SSRs and AFLP markers and 48 *L. temulentum* accessions from eight countries, Senda et al. (2004) found that genetic differentiation between the accessions was primarily a function of isolation distance and/or limited movement of seeds among countries. Based on the molecular markers and the *L. temulentum* accessions analyzed in this study, similarity among the accessions was not a function of the country of origin.

Festuca diversity and relationship with *Lolium*

The three tall fescue genotypes formed its own clusters with a similarity coefficient of 0.63. Tall fescue PI423078 was found to be quite different from KY31 and PI297901, the latter two having a similarity coefficient of 0.78. The two species, meadow fescue and tetraploid fescue each formed their independent clusters. However, tetraploid fescues were closer to tall fescue than meadow fescue. Tall fescue is an allohexaploid (2n = 6x = 42) with the genome constitution of PPG1G1G2G2. Meadow fescue (2n = 2x = 14, PP) is the donor of the P-genome while tetraploid fescue (2n = 4x = 28, G1G1G2G2) is the donor of the G1G2 genomes (Sleper 1985). Close relationship of tetraploid fescue with tall fescue might be due to sharing of the two genomes. The two meadow fescue accessions appeared very distinct with the lowest similarity among *Fescue* clusters. The *Fescue* clusters are distantly related to the Darnell ryegrass clusters (Fig. 1). Except L6, *Fescue* clusters have a Dice similarity of 0.26 to Darnel ryegrass. Similarities between L6 and meadow fescue entries are only 0.27. Mian et al. (2005) reported similar relationships among *Festuca* and *Lolium* species using EST-SSRs. Using RAPD and RFLP markers, Charmet et al. (1997) obtained similar results in a phenetic analysis of similarity matrices between cultivated *Festuca* and *Lolium*.

The results from bootstrap analysis generally supported the phylogenetic relationship deduced using the UPGMA. The clusters from UPGMA were very robust as revealed by bootstrap values (Fig. 1). The meadow- and the tetraploid fescue clusters were supported by 100% of bootstraps while the tall fescue cluster was supported by 99% of bootstraps. The clustering of *L. temulentum* accessions was supported by 100% of bootstraps.

Comparison of marker systems showed that both TF EST-SSRs and F × L Genomic SSRs were useful in assessing genetic diversity in *L. temulentum* accessions. The SSR markers identified in this study will be useful for future dissection of *L. temulentum* genotypes and in the analysis of F × L hybrids. *L. temulentum* has a short life cycle, is a diploid and is self pollinating, features not commonly shared with many forage species. It could be a useful model system for the study of basic biology of forage species and for genetic manipulation studies (Wang et al. 2002). It is closely related to major grass species in the *Festuca–Lolium* complex and orchardgrass and therefore could be used in the study of gene functions in related species. For example, *L. temulentum* was used to study the 'stay green' trait (controlled by the senescence induced degradation gene) of *F. pratensis* (Thomas and Stoddart 1975; Hauck et al. 1997). In that study, *F. pratensis* was crossed with *L. multiflorum* as a bridging species, and then to *L. temulentum* using both backcross and embryo-rescue techniques (Thomas et al. 1999). *L. temulentum* can also be exploited for the self-compatibility gene by introgressing the trait to the fescues, e.g. *F. pratensis* to create inbred lines (Yamada 2001).

Evaluation of *L. temulentum* accession L6

Leaf morphology and plant structure of L6 is different from some *L. temulentum* accessions (L28, L45) but similar to other (L40). Both seed shape and

size of L6 is distinct from other *L. temulentum* accessions (Fig. 2, Table 3) and closer to meadow fescue than *L. temulentum*. Molecular markers profile and seed characteristics indicate that L6 may be a meadow fescue and not a darnel ryegrass. This accession probably has been mislabeled in the past and further morphological and taxonomic studies are needed to confirm the true taxonomic classification of this accession. The molecular characterization of L6 in this study, however, demonstrates the power of DNA markers for identifying misclassified materials in germplasm collections.

We developed a set of microsatellite markers for *L. temulentum*. These markers were found effective for genetic diversity analysis of *Festuca* and *Lolium* species. The phenograms revealed that the *Lolium* accessions tested are genetically diverse particularly accession L6 (ABY-BA 8892.78) which did not cluster with other *L. temulentum* accessions. This accession appeared as a unique individual and merits further study. *L. temulentum* presents unique attributes as a model species for genetic and genomic studies in forage and turf grasses (Wang et al. 2005). This grass species is self-fertile, has a short life cycle, is a diploid, is easy to grow, and is closely related to members of the *Festuca* × *Lolium* complex. The diversity information from this study will be useful in future *Festuca* and *Festulolium* hybrid development.

Fig. 2 Seeds of eight *L. temulentum* accessions (L1, L5, L7, L26, L28, L40, L45, L47) randomly selected to represent the phylogenetic groups, L6 and a meadow fescue (MF)

Table 3 Seed characteristics of *L. temulentum* accessions randomly selected from each phylogenetic groups and L6 and compared with meadow fescue (MF)

Genotype	Seed length (mm)	Seed width (mm)	100 seed weight (g)
L1	6.32	2.13	0.873
L5	6.22	2.04	0.809
L6	5.96	1.51	0.216
L7	5.62	2.24	0.774
L26	5.56	2.05	0.744
L28	7.22	2.45	1.147
L40	5.80	2.36	1.198
L45	5.81	2.14	1.005
L47	6.78	2.74	1.861
MF	5.65	1.30	0.171
Mean	6.09	2.10	0.880
Std. dev.	0.55	0.42	0.486

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